

Rapid Nucleic Acid Detection of HIV-1 Using LAMP and RT-LAMP

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ABSTRACT

Background: A rapid, simple, cost efficient, nucleic acid based test for detecting HIV-1 in resource poor or field settings is highly desirable. Loop-mediated isothermal amplification is a novel method for amplifying DNA and RNA (LAMP and RT-LAMP, respectively) with high specificity, sensitivity, and simplicity. The method consists of incubating a mixture of the target gene, four or six different primers, Bst DNA polymerase, AMV reverse transcriptase (for RNA), and substrates. It's a one-step, isothermal reaction with a rapid outcome and no need for expensive equipment. In addition, visual detection is possible due to an increase in turbidity from the formation of magnesium pyrophosphate, a by-product of the reaction. This makes the LAMP method an attractive alternative for traditional PCR which requires dedicated equipment and can be time-consuming. For the first time, the LAMP and RT-LAMP methods have been applied for detection of HIV-1 and their sensitivity, specificity, and applicability for clinical specimens have been evaluated.

Methods: Primers targeted against highly conserved regions of HIV-1 were designed using software found on the Eiken WEB Page. For initial proof of concept, DNA was extracted from OM10.1 cells (QiaAmp DNA Blood Kit, Qiagen, MD) and RNA (QiaAmp Viral RNA Kit, Qiagen, MD) from a commercial source of Bal virus (ABI, MD). Subsequently, plasma from HIV-1 infected individuals, with known viral loads, was used either directly after heat treatment (99°C for 10 minutes) or after RNA extraction for amplification. To address specificity, HIV negative and HTLV-1 infected samples were used. A range of incubation times and temperatures were examined to determine optimum amplification conditions.

Results: As early as 30 minutes at 60°C, 10⁴ HIV-1 proviral copies were detected. For extracted RNA, detection was in the range of 10⁴ copies. Furthermore, we were able to amplify from as little as 5 µl of heat-treated plasma, with no RNA extraction. No amplification occurred using DNA, RNA or plasma from HTLV-1 infected or HIV negative samples.

Conclusions: The HIV LAMP or RT-LAMP assay is a cost effective, single tube assay that requires no sophisticated equipment. Due to its simplicity, the assay has potential as a diagnostic tool. The ability to amplify without extraction of nucleic acids, lower cost, and the rapid nature of the assay make this method highly attractive for use in resource poor settings and field applications.

BACKGROUND

Loop-Mediated Isothermal Amplification (LAMP)

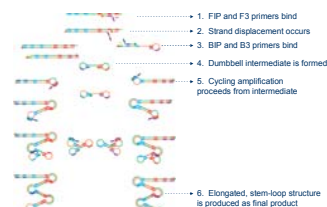
- Rapid nucleic acid detection method that can potentially be used as an alternative to RT-PCR for confirmation of HIV-1 infection
- One step amplification reaction that amplifies a target sequence with high specificity and sensitivity
- Can be used to detect DNA or viral RNA (RT-LAMP)
- Amplification occurs under isothermal conditions obviating the need for special equipment; Reactions can be carried out using a heat block
- Requires 4 or 6 (accelerated LAMP) primers specific for 6 different regions surrounding target sequence
- Detection of amplification can be observed visually due to accumulation of magnesium pyrophosphate, a by-product of the reaction, or through the addition of an intercalating dye
- Detection of amplified LAMP product has been observed as early as 15 minutes

LAMP/RT-LAMP Primers

- LAMP primers are designed using 6 arbitrary regions within the target sequence, termed F1, F2, F3, B1, B2, and B3
- Primers:
 - F3 and B3:** Forward outer and Backward outer primers; Contain the F3 and B3 sequences, respectively
 - F1P and B1B:** Forward inner and Backward inner primers; Contain F2/B2 sequence and complement to F1/B2 (F1/B1C)
 - Loop F and Loop B:** Bind to loops of 'dumbbell' intermediate formed during amplification; Contain complementary sequence to single stranded region between F1/B1 and F2/B2



Basic Principle of LAMP



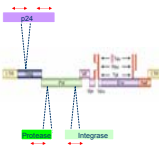
OBJECTIVES

- Compare sensitivity of LAMP to PCR
- Determine specificity of LAMP
- Determine whether RT-LAMP can be used to detect viral RNA
- Determine necessity of RNA extraction for the detection of HIV-1 in plasma samples
- Evaluate timing of LAMP/RT-LAMP reaction

MATERIALS AND METHODS

Design of HIV-1 LAMP Primers

- Primers were designed using primer designing software available on the Eiken website (<http://primerexplorer.jp/e/>)
- Currently, 4 different primer sets have been designed in our lab directed against regions within p24, protease, and integrase genes (data will only be shown for 1 of the 2 sets of p24 primers)



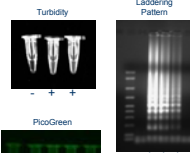
Denotes approximate location of target sequence against which primers were designed

Procedure Summary

- Samples**
 - Extracted DNA from HIV-1 infected cell line- OM-10.1 (Negative control- HUT102 DNA)
 - Extracted RNA from lab-adapted HIV-1 strain- Bal, or patient plasma (Negative control- HUT102 RNA or negative plasma)
 - Heat treated patient plasma (Negative control- negative plasma)
- Sample Preparation**
 - DNA extraction- QiaAmp DNA Blood Mini Kit (Qiagen)
 - RNA extraction- QiaAmp Viral RNA Mini Kit (Qiagen)
 - Heat treatment- Plasma was heated at 99°C for 10 minutes
- Master Mix Preparation**
 - Contains: Bst DNA polymerase, dNTPs, 6 LAMP primers, reaction buffer, AMV reverse transcriptase (RT-LAMP only)
 - 10 µl of sample was added to each reaction tube, for a total reaction volume of 25 µl (unless otherwise stated)
- Amplification**
 - Performed in heat block or thermocycler at 58 or 60°C, depending on primer set
 - Samples were held at reaction temperature for 60 minutes (unless otherwise stated)
- Detection (See figure below)**
 - Amplification was observed visually by turbidity or addition of intercalating dye (PicoGreen)
 - Positive reaction was confirmed by running amplified samples on an agarose gel

Detection of Amplified Product

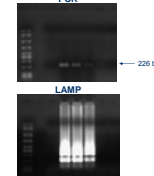
- Visual detection**
 - Positive reaction can be identified visually due to turbidity (caused by accumulation of magnesium pyrophosphate by-product)
 - Visual detection can be facilitated by addition of intercalating dye, such as PicoGreen
- Agarose gel**
 - Positive LAMP reaction can be verified by agarose gel due to the characteristic laddering pattern observed
 - The various size bands noted on the gel are the result of replicating intermediates and the elongated, stem-loop final product



RESULTS

Sensitivity of LAMP vs. PCR

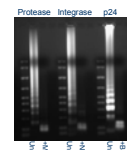
- Methods:**
 - PCR and LAMP were performed on extracted DNA from an HIV-1 infected cell line (OM-10.1)
 - QiaAmp DNA Blood Mini Kit (Qiagen) was used for DNA extraction
 - LAMP- PCR was performed using 6 LAMP primers directed against a specific region of p24
 - PCR- F3 and B3 outer LAMP primers were used
- Results:**
 - PCR and LAMP show comparable sensitivity- Amplification was observed down to 100 copies using both methods
 - Amplification of HTLV-1 DNA was not observed (data not shown)



Ultimate sensitivity of LAMP primers to DNA is yet to be determined, but experiments have yielded positive results between 10-1000 copies/tube

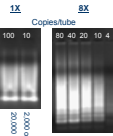
Specificity of LAMP Amplification

- Methods:**
 - LAMP was performed on extracted OM-10.1 DNA (100 copies) using 3 different primer sets
 - Restriction sites located within the target sequences (amplified by the LAMP primers) were identified
 - Amplified LAMP products were digested with specific restriction enzymes and results were compared to undigested product
- Results:**
 - All 3 primer sets amplified 100 copies of HIV-1 DNA, but not HTLV-1 (data not shown)
 - Restriction digest yielded expected bands, indicating specificity



Increasing Reaction Volume Sensitivity Will increasing reaction volume equate to increased sample sensitivity?

- Methods:**
 - LAMP was performed on various dilutions of extracted OM-10.1 DNA using protease primers
 - Sample volume was added to reaction tube as in previous experiments (1X) or increased 8-fold (8X)
- Results:**
 - Sensitivity remained approximately 10 copies/tube in 8x reaction, but overall sample sensitivity increased 8-fold (2,000 copies/ml to 250 copies/ml)

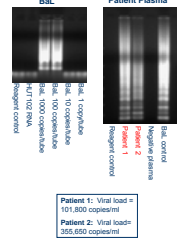


CONCLUSIONS

- LAMP has similar sensitivity to PCR
- The LAMP reaction is highly specific, amplifying only the target sequence recognized by the LAMP primers
- LAMP sensitivity can be increased by increasing the sample volume
- RT-LAMP can detect HIV-1 present in patient plasma samples, both from extracted RNA and heat treated plasma
- LAMP can amplify HIV-1 DNA as early as 15 minutes, but maximum sensitivity (10 copies) is reached after 30 minutes
- Detection of viral RNA by RT-LAMP can be observed as early as 30 minutes
- Sensitivity of LAMP/RT-LAMP needs to be further evaluated for patient samples

Can RT-LAMP be used to detect viral RNA?

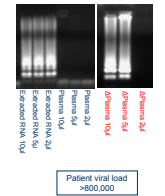
- Methods:**
 - Reverse transcriptase was added to reaction mixture, containing protease specific primers
 - RT-LAMP reaction was carried out on varying concentrations of RNA extracted from lab-adapted strain of HIV-1 (Bal) or plasma samples from HIV infected patients
 - QiaAmp Viral RNA Mini Kit was used for RNA extraction (Qiagen)
- Results:**
 - RT-LAMP successfully amplified from Bal, RNA and RNA from plasma samples
 - All 4 primer sets were able to amplify from both plasma samples, except integrase which only detected 1 patient sample (data only shown for protease primers)



Sensitivity of LAMP primers to RNA is yet to be determined, but initial experiments have yielded positive results between 100 and 1000 copies/tube

RT-LAMP Is RNA extraction necessary for detection of HIV in plasma samples?

- Methods:**
 - RT-LAMP was performed on RNA extracted from plasma samples of HIV negative and seropositive patients, using p24 primers
 - To determine the necessity of nuclear extraction, untreated and heat treated (Δ) plasma samples were also tested
- Results:**
 - Amplified product was detected in both extracted RNA and heat treated samples from a HIV-1 infected patient sample
 - Amplified product was not detected in untreated and HIV negative (data not shown) plasma samples



Timing of LAMP/RT-LAMP How quickly can amplified product be detected?

- Methods:**
 - LAMP and RT-LAMP were performed on extracted OM-10.1 DNA and Bal, RNA, respectively, using p24 primers
 - Reactions were allowed to proceed for 15, 30, or 60 minutes
- Results:**
 - Amplified LAMP product was observed as early as 15 minutes, but maximum sensitivity was reached at 30 minutes (10 copies)
 - RT-LAMP amplification was observed as early as 30 minutes

